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Short-Form Paper

Rifabutin is inactivated by *Mycobacterium abscessus* Arr

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Abstract

Mycobacterium abscessus **exhibits** ***arr*** **(ADP-ribosyltransferase)-dependent rifampicin (RIF) resistance. In apparent contrast, rifabutin (RBT) has demonstrated promising activity in** ***M. abscessus*** **infection models implying that RBT might not be inactivated by Arr. RBT susceptibility testing of** ***M. abscessus Δarr*** **revealed a strongly decreased minimal inhibitory concentration (MIC). Our findings therefore suggest that the efficacy of RBT might be enhanced by rendering RBT resilient to Arr-dependent modification or by blocking** ***M. abscessus*** **Arr activity.**

The incidence and prevalence of pulmonary disease caused by infections with non-tuberculous mycobacteria (NTM) is increasing worldwide. NTM-pulmonary disease requires a long-lasting therapy of up to two years which however has a success rate of less than 50% (1, 2). *Mycobacterium abscessus* complex (MABSC) comprised of the three subspecies *Mycobacterium abscessus* subsp. *abscessus*, *Mycobacterium abscessus* subsp. *bolletii* and *Mycobacterium abscessus* subsp. *massiliense* (3) is characterized by its extreme drug resistance to both broad-spectrum drugs and tuberculosis-specific drugs (4, 5). Consequently, treatment options are limited and novel drugs are urgently needed. Unfortunately, *M. abscessus* drug-screens revealed a low hit rate limiting the development of drugs with a new mode of action (6, 7). However, repurposing of existing drugs might offer new treatment options (8).

Rifamycins act as inhibitors of the DNA-dependent RNA polymerase (9, 10). Rifampicin (RMP), although highly active against *Mycobacterium tuberculosis* (critical concentration in MGIT 1.0 mg/L) (11) shows very poor growth inhibition of *M. abscessus* (MIC 128 mg/L) due to the drug-modifying activity of its ADP-ribosyltransferase Arr_{MAB} (12). Arr enzymes modify the C_{23}-hydroxyl group within the rifamycin core structure (13). Deletion of arr_{Mab} in *M. abscessus* has been shown not only to decrease the MIC for RMP but also for two other rifamycins, i.e. rifaximine (RFX) and rifapentine (RPT) (12).

Recently, the RMP analogue rifabutin (RBT) was identified in an *in vitro M. abscessus* drug-screen and verified to be active against a variety of *M. abscessus* strains (MIC ≈ 3 mg/L) (14). Subsequently, RBT proved to be efficacious in *in vitro* and *in vivo* models of *M. abscessus* infection while RMP lacked activity (15, 16). The antibacterial *in vitro* and *in vivo* activity suggests that RBT unlike the other rifamycins might not be a substrate for Arr_{MAB} although MIC of RBT for mycobacterial pathogens without *arr* [*M. tuberculosis* (critical concentrations RBT = 0.1 mg/L vs RMP 1.0 mg/) and *M. avium*] are generally lower than for RMP (11).

We conducted RBT susceptibility testing (**Supplemental Material**) of *M. abscessus* ATCC 19977T, its isogenic *Δarr* deletion mutant and the complemented mutant Δarr_{MAB} pMV361_arr_{MAB} (12) to resolve this issue (**Table 1; Supplemental Table S1**). RFX and RMP served as a control. *M. abscessus* (wt) showed a RBT MIC of 4 mg/L, while MICs for rifaximine (RFX; 32 mg/L) and particularly for RMP (128 mg/L) were considerably higher. The *M. abscessus* Δarr_{MAB} mutant exhibited increased susceptibility to RMP, RFX and particularly towards RBT (**Table 1**). The $\log_2$-transformed relative resistance ratios (RRR) $MIC_{wt}/MIC_{\Delta arr}$ for RBT, RFX and RMP were 18, 4 and 8, respectively. The MICs of other drug classes [amikacin (AMK), tetracycline (TET)] were not affected by the *arr* genotype (RRR = 0 – 1). The wild-type MICs towards the rifamycins were restored in the complemented deletion mutant. The low RBT MIC of the *M. abscessus Δarr* deletion mutant indicates that RBT is a substrate for Arr. Furthermore, the lower RRR for RMP as compared to the RRR for RBT (8 vs. 18) suggests that resistance determinants other than Arr might selectively inactivate RMP but not RBT.

RMP monooxygenases (Rox) are present in a wide variety of environmental bacteria and are associated with decomposition of RMP (17). A corresponding rifamycin resistance mechanism has also been proposed for *M. abscessus* (18). Using Rox genes *of Streptomyces venezuelae* (Sven_0481) and *Nocardia farcinica* (Nfa_35080) as a query in a BlastP search putative *M. abscessus* orthologues MAB_0857, MAB_3484 and MAB_1496c were identified **(Supplemental Table S2**). Of these, MAB_1496c has recently been demonstrated to be a member of the *M. abscessus* resistome due to its involvement in tetracycline oxygenation (19). Single and multiple unmarked deletion mutants (**Figure 1; Supplemental Figure S1**) in *M. abscessus* ATCC 19977T and *M. abscessus Δarr* were constructed by allelic replacement with suicide vectors containing PCR-amplified flanking regions of the target genes (**Supplemental Table S3**) and apramycin-positive and *katG* (INH^S)-negative selection markers (20, 21) (**Fig. 1**). Mutants were confirmed by PCR and Southern blot analysis (**Supplemental Figure S1**) and tested for rifamycin, tetracycline and amikacin susceptibility (**Table 1**). Exploratory investigations indicated that neither individual deletion of MAB_0857, MAB_3483 and MAB_1496c nor combined deletion of MAB_0857 and MAB_3483 in *M. abscessus* ATCC 19977T affected the RBT,

RFX, RMP and AMK MIC, respectively. Likewise, deletion of these genes in *M. abscessus* Δarr_{MAB} did not alter susceptibility towards these antibiotics as compared to *M. abscessus* Δarr_{MAB} indicating that these genes do not affect rifamycins. Deletion of MAB_1496c, encoding monooxygenase TetX decreased the tetracycline MIC 16 -32-fold, while rifamycin MICs were not affected (for the MIC of the most advanced strains see **Table 1**).

So far, our investigations have not identified genetic determinants responsible for selective RMP modification. A phenotypic screen of a genome wide transposon mutant library might be the most efficient way to identify those resistance determinants (22, 23). However, our findings on RBT susceptibility of the *Δarr* mutant are of major importance for future drug development. The RRR of about 250.000 (log_2=18) clearly shows that RBT is a substrate of Arr which in turn suggests that the potency of RBT against *M. abscessus* might be improved by either Arr-inhibitors or by RBT modification. Regimens co-applying drugs in conjunction with inhibitors of drug-modifying enzymes have been developed to restore antibiotic activity and are widely used in clinics (24-26). Similarly, Arr-inhibitors might be beneficial to establish a rifamycin-based treatment for *M. abscessus* infections. Alternatively modifications of the drug itself might render RBT resilient towards Arr. E.g. substitution of the acetyl moiety by a bulky residue at position C_{25} rendered RMP partially resistant towards Arr-modification (12, 27, 28). We envision that these approaches may improve treatment options and outcomes of pulmonary disease caused by the opportunistic pathogen feared for its high-level drug resistance, *M. abscessus*.

Authors contributions

Conceptualization: P.Sa., B.S., A.R.; investigations: D.S., P.Se, B.B., M.M., A.R.; writing – original draft preparation D.S., P.Sa; writing – review and editing, P.Sa. All authors have read and agreed to the published version of the article.

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Conflict of interest

The authors declare no conflict of interest.

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200 **Table 1: Minimal Inhibitory Concentration (MIC)* and Relative Resistance Ratios (RRR)** of strains**

Strain	RBT		RFX		RMP		TET		AMK	
	MIC	RRR	MIC	RRR	MIC	RRR	MIC	RRR	MIC	RRR
M. abscessus	4	/	32	/	128	/	32	/	1	/
Δarr$_{MAB}$	1.1x10^{-5}	18	1.5	4	0.38	8	24	0	1	0
Δarr$_{MAB}$ pMV361-*arr*$_{Mab}$	4	0	16	1	64	1	16	1	1	0
Δarr$_{MAB}$ *ΔMAB_0857* *ΔMAB_3483*	4.6x10^{-5}	16	1.5	4	0.25	9	24	0	1	0
Δarr$_{MAB}$ *ΔMAB_1496c*	4.6x10^{-5}	16	1.5	4	0.19	9	1	5	1	0

201 *MIC (median of 6 replicates) in mg/L; **Relative Resistance Ratio (RRR) $\log_2 MIC_{wt}/MIC_{comparator}$

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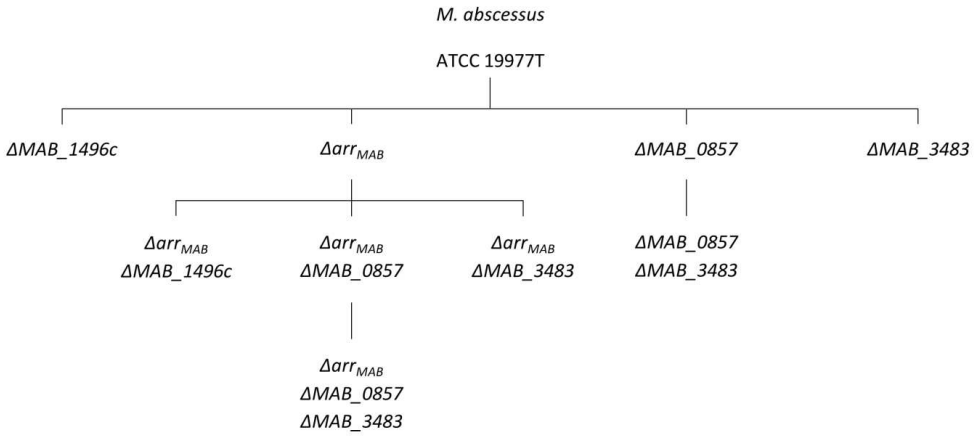

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206 **Figure 1**

207 Genealogy of strains

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